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**VEGFC reduces glomerular albumin permeability and protects against alterations in  
VEGF receptor expression in diabetic nephropathy**

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## Abstract

Elevated levels of vascular endothelial growth factor (VEGF)A are thought to cause glomerular endothelial cell (GEnC) dysfunction and albuminuria in diabetic nephropathy. We hypothesized that VEGFC could counteract these effects of VEGFA to protect the glomerular filtration barrier and reduce albuminuria. Isolated glomeruli were stimulated *ex vivo* with VEGFC, which reduced VEGFA- and type II diabetes-induced glomerular albumin permeability (Ps'alb). VEGFC had no detrimental effect on glomerular function *in vivo* when over-expression was induced locally in podocytes (podVEGFC) in otherwise healthy mice. Further, these mice had reduced glomerular VEGFA mRNA expression, yet increased glomerular VEGF receptor heterodimerization indicating differential signalling by VEGFC. In a model of type I diabetes, induction of podocyte VEGFC over-expression reduced the development of hypertrophy, albuminuria, loss of GEnC fenestrations and protected against altered VEGF receptor expression. In addition, VEGFC protected against raised Ps'alb by endothelial glycocalyx disruption in glomeruli. In summary VEGFC; reduced the development of diabetic nephropathy; prevented VEGF receptor alterations in the diabetic glomerulus; promoted both glomerular protection and endothelial barrier function. These important findings highlight a novel pathway for future investigation in the treatment of diabetic nephropathy.

## Introduction

Systemic endothelial dysfunction is an initiating step in the development of vascular damage in diabetes (1; 2) and is associated with microalbuminuria (urinary albumin secretion, 30-300mg/day) (3). It is widely accepted that microalbuminuria indicates disruption of the glomerulus and is the earliest clinically detectable indicator of incipient diabetic nephropathy (DN) (4). Glomerular endothelial cells (GEnC) restrict the passage of protein across the highly specialized glomerular capillary wall via the luminal facing endothelial glycocalyx (eGLX), which consists of proteoglycans, glycosaminoglycans, glycoproteins and trapped soluble plasma proteins. GEnC have trans-cellular fenestrations that form in attenuated areas of GEnC cytoplasm which constitute 20-50% of the entire endothelial cell surface (5). Fenestrae are necessary for the high permeability of the glomerulus to water and small solutes (5). However, fenestrae are covered by eGLX (6) which is known to regulate vascular permeability (7-10), by limiting the passage of charged macromolecules (11).

Vascular endothelial growth factor (VEGF)-A is highly expressed by podocytes in the glomerulus (12) and provides essential maintenance signals for GEnC including those for survival and regeneration (13), maintaining fenestrations (14) and regulating solute flux and protein passage (15; 16). Systemic and glomerular vessels become more “leaky” in pathological conditions where VEGFA bioavailability is increased, such as cancer, retinal disease (17) and early DN (18; 19). Blockade of VEGFA in these experimental models reduces vascular permeability (20; 21), including proteinuria in DN (6; 22). However, clinical anti-VEGFA therapies (23-25) can cause proteinuria and hypertension (26) confirming that some VEGFA is necessary for endothelial maintenance. Therefore, strategies which block adverse effects of excessive VEGFA, but do not remove it completely would be attractive for DN.

VEGFC, a lymphangiogenic growth factor, is also expressed by podocytes (27), despite an absence of lymphatic vessels in glomeruli. VEGFC typically signals through VEGFR3, predominantly expressed in lymphatic endothelial cells (LEC), but can induce similar “maintenance” signals as VEGFA in vascular endothelial cells (28) acting through VEGFR3, VEGFR2 or heterodimers of both (29). VEGFC can also induce fenestration formations in the vascular endothelium, but to a lesser extent than VEGFA (30).

VEGFA predominantly signals through phosphorylation of VEGFR2 (31). We have shown that VEGFA induces transient VEGFR2 phosphorylation after 2min in human conditionally immortalized (ci)GEnC (32). VEGFC also induced VEGFR2 phosphorylation in these cells, maximally at 30min, but did not induce VEGFR3 phosphorylation. VEGFC induced a correspondingly slower rise in intracellular calcium than VEGFA and did not result in VE-Cadherin phosphorylation. We hypothesise that such differences in intracellular events underlie our observation that VEGFA increases permeability of ciGEnC monolayers to protein while VEGFC reduces protein permeability and blocks the effect of VEGFA (32). Additionally, VEGFC enhances the eGLX layer *in vitro*, in contrast to VEGFA which increases eGLX shedding (32; 33).

Together, these data suggest that VEGFC may be able to ameliorate albuminuria by reducing the permeability of GEnC to protein, whilst retaining high water permeability. Thus, the effect of VEGFC on raised glomerular albumin permeability was assessed under various conditions and VEGF-C receptor signalling was explored. Finally, the mechanism of VEGFC action on GEnC barrier properties were investigated with a specific focus on the eGLX.

## Research design and methods

*Glomerular endothelial cell culture:* Human conditionally immortalised glomerular endothelial cells (ciGEnC) were maintained and propagated in culture as described (34). ciGEnC, stably transfected with GFP-tagged VEGFR2 (ciGEnC/GFP-VEGFR2) (a kind gift from Prof Harry Mellor, University of Bristol) were cultured similarly.

*Recombinant proteins:* Human recombinant VEGFA and VEGFC (R&D Systems) were reconstituted as previously and used at final concentrations of 1nM (42 ng/ml) and 10nM (210 ng/ml) respectively as previously (32).

*Antibodies:* All primary and secondary antibodies that were used and their dilutions are described in table 1 (supplementary material).

*Immunofluorescence:* Immunofluorescence was carried out on fresh frozen mouse kidney sections as previously (35) and on GEnC as previously (33). The integrated density of glomerular VEGFC was semi-quantified using Image J (15 glomeruli per animal) and expressed as fold change to littermate control mice (LMC). For cellular VEGFR3 quantification, images were acquired using the InCell Analyzer 2200 system (GE Healthcare) as previously (36). In brief, analysis was performed using InCell Analyzer 1000 Workstation Multi-target analysis algorithms using phalloidin to define the cell and DAPI to segment the nuclei. VEGFR3 is reported as mean fluorescence intensity per cell.

*Western blotting:* ciGEnC, tissue, sieved glomeruli and flow-through were lysed in RIPA buffer containing phosphatase and protease inhibitors. Samples were quantified (bicinchoninic acid assay, Pierce; Thermo Fisher Scientific) and Western blotted.

*RNA extraction and PCR:* RNA was extracted from isolated mouse glomeruli using a RNeasy Mini kit (Qiagen) according to manufacturer's instructions. Primer sequences were predesigned,

selected from <http://pga.mgh.harvard.edu/primerbank/> and optimized as previously described (33). Human GAPDH primers were used as previously (33), with other primer pairs described in table 2 (supplementary material). QPCR was carried out as previously described (33).  $2^{-\Delta\Delta CT}$  (relative fold change) was calculated from the CT values generated.

*VEGFR3 Immunoprecipitations (IP):* Mouse lungs and glomeruli were freshly harvested in ice cold PBS containing 100 $\mu$ M Na<sub>3</sub>VO<sub>4</sub> and protease inhibitor cocktails (1:100, Sigma). These tissues were then treated with VEGFA and VEGFC or vehicle at 37°C for 2, 15 or 30min. PBS was removed and samples resuspended in 100 $\mu$ l NP40 lysis buffer, then pre-cleared. Equal quantities of protein, made to 500 $\mu$ l, were incubated at 4°C for 18h with 1.5 $\mu$ g/100 $\mu$ g protein of anti-mouse VEGFR3 (ALF4) (eBioscience). Subsequently, 60 $\mu$ l of equilibrated A/G PLUS-agarose beads (Santa Cruz) were added to each sample and incubated at 4°C for 6h. Beads were centrifuged and supernatant samples were taken for immunoblotting. Thirty microlitres of IP samples and equal protein concentrations of supernatant and total protein were loaded onto an SDS PAGE gel and Western blotted.

*Podocin rtTA, Tet-O-VEGFC (podVEGFC) mice:* Podocin rtTA (podrtTA) mice expressing the transgene exclusively in podocytes (37) were a kind gift from Prof Jeffrey Kopp, NIH (FVB/mixed). Tet-O-VEGFC mice (FVB/N), containing full length mouse VEGFC were used as previously described (38). Heterozygous podrtTA mice were crossed with heterozygous Tet-O-VEGFC mice to obtain mice heterozygous for both transgenes (podVEGFC) and LMC mice. Animals were kept according to the “Guidelines on the Use of Animals in Research,” and the number of animals used was kept to a minimum. Mice were housed in a pathogen-free environment at 21°C, with a 12-h light-dark cycle, and all received a standard laboratory animal diet (Beekey Feeds) and water ad libitum.



*Animal genotyping:* Ear notches were digested in 20µl digestion buffer (1X PCR buffer (supplied with Hotmaster taq) 0.0045% NP40, 0.0045% Tween 20, 1mg/ml proteinase K). Samples were digested for 2hs at 60°C, then 15min at 95°C. PCR for podrtTA was carried out as originally described (37). For Tet-O-VEGFC, primers are described in table 2 (supplementary material). The PCR reaction (total volume 13µl) contained 6.5µl Mastermix buffer (Amplitaq Gold 360, Applied Biosystems) and 0.5µM primers. PCR cycle conditions were as follows; 1 cycle at 95°C for 10min, 42 cycles of 1 cycle at 95°C for 30sec, 68°C 30sec, 72°C for 60sec and 72°C for 7min.

*Induction of podocyte VEGFC overexpression in transgenic mice.* Expression of podocyte VEGFC was induced by doxycycline (2 mg/ml) in the drinking water with sucrose (5% wt/vol) as previously shown (37). Doxycycline was replaced every 3-4 days and protected from light at all times. If mice did not drink the doxycycline water they received chow supplemented with 625mg/kg doxycycline (Harlan Laboratories UK), observed to increase VEGFC mRNA expression to a similar extent (0.9-fold change VEGFC mRNA expression compared to doxycycline supplemented water, data not shown). Some animals were given sucrose alone. Urine samples were collected weekly in metabolic cages with environmental enrichment up to 6h at a time.

*Type II diabetic mice:* 12-14 wk old male C57BLKsJ-db/db (db/db) mice (Harlan Laboratories, UK) were used as a model of severe spontaneous Type II diabetes. Age matched, male C57BLKsJ-db/+ (db/+) lean mice were used for comparison. mRNA was extracted from enriched glomeruli from 7wk old db/db and db/+ mice. Urinary albumin creatinine ratio (uACR) and glucose levels were measured as below.

*Induction of Type I Diabetes:* Diabetes was induced in 8-10wk podVEGFC and LMC mice using a low-dose (50mg/Kg/day over 5 consecutive days) streptozotocin (STZ) induction protocol as previously (39) (as advised by DiaComp <https://www.diacomp.org/>). Mice were checked for adverse effects following dosing; animals that demonstrated dehydration (including weight loss, slack skin or bloating due to constipation-revealed by post-mortem in the first few diabetic mice) were supplemented with 'mash' and DietGel with subcutaneous 0.9% saline injections as necessary. Blood glucose levels were monitored weekly from a tail-tip blood droplet using an Accu-check (Aviva) blood glucose meter. Animals with blood glucose above 15mmol/L 2wk after injections were included in the study. Body weight was monitored regularly. Mice received doxycycline from 0wk (prevention) or 4wk (rescue) after STZ administration. Urine was collected as previously, although as animals became more polyuric the collection time was reduced to minimise water weight loss and stress.

*Glomerular albumin permeability (Ps'alb) assay:* This assay was developed and extensively characterized previously(40). In brief, mice were anaesthetised and the kidneys perfused with 4% BSA (RingerBSA), followed by 36.5µg/ml Octadecyl rhodamine B chloride (R18) (ThermoFisher Scientific) to label cell membranes, then 30µg/ml AlexaFluor (AF)488-BSA (labelled albumin) (Life technologies). Glomeruli were isolated from kidney cortex by graded sieving. For glomeruli treated *ex vivo*, glomeruli loaded with AF488-BSA were held on ice until treated with VEGFA, VEGFC, both VEGFA and VEGFC, or an equal volume of Ringer-BSA (vehicle) for 1h at 37°C. Individual glomeruli were transferred to a Nikon TIE inverted confocal microscope and trapped before the perfusate was switched from 30µg/ml AF488-BSA to 30µg/ml unlabelled BSA. The rate of decrease in fluorescent intensity within capillary loops was quantified and from this apparent albumin solute permeability (Ps'alb) was calculated(40) :

$$P_{salb} = -kR/2$$

*In vivo eGLX disruption:* We have shown previously that chondroitinase and hyaluronidase given acutely, i.v. 30min before sacrifice raises Ps'alb significantly and causes a reduction in eGLX coverage(40) . In the same cohort of animals, glomeruli were isolated and incubated in VEGFC as described above and Ps'alb measured and compared. Further, chondroitinase and hyaluronidase were administered chronically, via an osmotic minipump (Charles River UK Ltd, Kent, UK, 0.25µl/h), cannulated to the jugular, for a total of 2 wk (0.087 mU/g chondroitinase and 15 mU/g hyaluronidase, or vehicle over a 24h period, equivalent to 0.004mU/h chondroitinase and 0.625U/h hyaluronidase). In the final week a VEGFC intervention was given to half of the enzyme treated mice i.p daily (100 ng/g body wt as previously(40) ). Glomeruli were isolated and Ps'alb was measured.

*Electron microscopy:* Mice were whole body perfusion fixed with Glutaraldehyde containing Alcian blue. Electron micrographs were taken using a Technai 12 electron microscope and images analysed as previously (39).

*Urinary albumin creatinine ratio (uACR):* Mouse uACR were quantified as previously described (39) and expressed as log fold change to baseline.

*Glomerular isolation:* To achieve a >97% pure population of isolated glomeruli, Dynabeads were cardiac perfused as originally described by Takemoto et al (43) and placed in RNA later (Invitrogen). Alternatively, to obtain a glomerular enriched population, kidneys were mashed and glomeruli isolated through graded sieving and collected from 75µm<sup>2</sup> pore sieves for protein or mRNA extraction. To demonstrate glomerular enrichment, kidney cortex, flow-through and sieved glomeruli were collected and lysates were extracted, quantified and Western blotted as

above. Blots were probed with anti-nephrin, anti-CD13 and anti actin antibodies (table 1, supplementary materials).

*Proximity Ligation Assay (PLA):* Fresh-frozen mouse kidney sections were fixed in 4% PFA and permeabilised using 0.1% Triton-X. Samples were then treated with the Proximity Ligation Assay Kit (Olink Bioscience) according to the manufacturer's instructions. In brief, primary antibodies or matched concentration IgG controls in various combinations were incubated together overnight (see table 1, supplementary materials for details), followed by incubation with goat MINUS and rabbit PLUS probes. Nuclei were counterstained using DAPI (1:1000 diluted in PLA kit 0.01x wash buffer B). Images were quantified, by a blinded analyser using ImageJ, by counting the number of punctate dots through z-stack of images and normalising to the number of nuclei within the field of view. Three representative z-stacks from each sample were analysed.

*Periodic acid-Schiff's (PAS) staining of mouse kidney sections:* Paraffin embedded mouse kidney sections were stained using a PAS kit (Sigma-Aldrich) according to the manufactures' instructions. Six glomerular images were analysed per mouse and quantified as previously (40).

*Picrosirius Red Staining:* Mouse tissue sections were hydrated followed by counterstaining in a 0.1% (w/v) Direct Red 80 powder (Sigma) in a 1.3% saturated aqueous solution of picric acid (VWR Chemicals) for 90min at room temperature, then dehydrated and mounted. Images were taken using bright field (to indicate collagen I and III) and polarizing lens' (to distinguish between type I (yellow-orange) and type III (green-white) newly laid collagen fibers).

*Statistical analysis:* All statistical analysis was carried out on a minimum of 3 separate experiments. In Ps' alb experiments, data are presented as means of measurements from glomeruli to show the spread of data points, but for statistical purposes the means from individual animals were used. In experiments with multiple treatments, a one-way ANOVA was

used with Bonferroni's multiple comparison post hoc test, unless indicated otherwise. A p value of less than 0.05 was considered statistically significant. Error bars indicate the standard deviation.

## Results

*VEGFC prevents the increase in glomerular albumin permeability caused by VEGFA and type II diabetes and is cytoprotective for GEnC.*

To test whether VEGFC could protect against the effects of raised VEGFA, an *ex vivo* isolated glomerular Ps'alb assay was utilized that we recently published(40) . VEGFA significantly increased Ps'alb in isolated glomeruli as anticipated in FVB/mixed background (fig 1A) and db/- (lean) mice (fig 1B). Importantly, this effect was prevented by VEGFC, yet VEGFC alone did not change Ps'alb. To understand whether VEGFC could rescue albumin permeability induced by diabetes, glomeruli were isolated from db/db (type II diabetic) mice and stimulated with VEGFC *ex vivo* as above. Ps'alb in diabetic glomeruli was significantly raised, as anticipated, whereas it was significantly rescued by VEGFC (fig 1C). Db/db mice were 12-14 wk old, had significant proteinuria (fig 1D) and were hyperglycemic (fig 1E). We confirmed that there was a significant increase in VEGFA mRNA expression in enriched glomeruli from db/db mice (fig 1F). We also confirmed that VEGFC was cytoprotective for GEnC, an important protective effect of VEGFA. Using a trypan blue viability assay we determined that GEnC were protected from death (serum free, volume reduced media) by VEGFC over 24h in a similar manner to VEGFA (supp fig 1A). This was associated with increased Akt phosphorylation over time by VEGFC and VEGFA (supp fig 1B), suggesting that VEGFC promotes survival in GEnC in a similar manner to VEGFA *in vitro*.

### *Characterization of podVEGFC mice*

To determine whether VEGFC could be protective *in vivo*, we generated a podVEGFC mouse (fig 2A). Firstly, glomeruli were Dynabead isolated from podVEGFC and LMC mice after receiving doxycycline for 3wk. Quantitative PCR revealed a significant increase in

normalized VEGFC glomerular mRNA expression in podVEGFC mice (fig 2B) This was supported by immunofluorescence showing co-localisation of VEGFC with nephrin (C) in podVEGFC mice. Semi-quantification demonstrated significantly increased glomerular protein expression in podVEGFC mice (fig 2D). Levels of VEGFC mRNA were similar in glomeruli isolated by Dynabead and sieving protocols (data not shown), therefore sieving was used to isolate glomeruli hereafter.

*VEGFC has no detrimental effect on glomerular function*

Glomerular parameters in podVEGFC mice were compared to LMC. There was no renal hypertrophy (fig 2E), mesangial matrix expansion (fig 2Fi&G) or ultrastructural changes in GMB thickness, podocyte foot process width, slit diaphragm length (fig 2Fii&Hi), fenestration density (fig 2I) or in uACR (fig 2J) up to 8wk after induction of VEGFC expression. Together, these results demonstrate that VEGFC does not significantly affect glomerular structure or function over prolonged periods of exposure, in contrast to VEGFA (44).

*Glomerular VEGFC expression affects VEGF signalling*

Glomerular sieving successfully enriched the glomeruli population (supp fig 2). In podVEGFC mice there was a trend towards a reduction in VEGFR2 in the glomerular enriched fraction (fig 3A), but no significant reduction in VEGFR3 mRNA expression (fig 3B). VEGFA mRNA expression was significantly reduced (fig 3C). In non-permeabilised cells both VEGFA and VEGFC reduced cell surface VEGFR3 expression at 2min (fig 3D), whilst total VEGFR2 and VEGFR3 expression was unaffected by VEGFA or VEGFC at 2min (supp fig 3). VEGFR3 and VEGFR2 heterodimerisation by VEGFC, as reported previously elsewhere (29; 45), was investigated *ex vivo* in isolated glomeruli using immunoprecipitation. VEGFR2 was not pulled down by VEGFR3 in response to VEGFC (fig 3E). However, heterodimers were formed in

response to VEGFC in mouse lungs as anticipated (fig 3F). Nilsson *et al* demonstrated that a proximity ligation assay (PLA) was more sensitive to detect VEGFR2/VEGFR3 heterodimerisation than immunoprecipitation (29). Thus, a PLA was used on fresh frozen sections from podVEGFC and LMC mice (fig 3G). Optimisation, by replacing primary antibodies with matched concentration IgG, demonstrated specificity of punctate staining (supp fig 4). There was a significant increase in punctate fluorescent events, indicating heterodimerization, throughout the Z stacks of each glomerular cell in podVEGFC mice compared to LMC mice (fig 3H).

*Early glomerular VEGFC expression reduces the development of DN*

Endothelial dysfunction develops early in diabetes and manifests as microalbuminuria in the kidney. We have shown that VEGFC ameliorates raised Ps'alb associated with type II diabetes in isolated glomeruli. To understand whether VEGFC could prevent albuminuria in DN, doxycycline was administered before STZ injections (fig 4A). Diabetic mice (D-) were hyperglycemic (fig 4B), had reduced body weight (fig 4C) and increased urine output (fig 4D), as anticipated. D-podVEGFC mice did not have significant renal hypertrophy (fig 4E) and had a significantly reduced fold change in uACR compared to D-LMC mice (fig 4F, please refer to supp fig 5A for absolute values), which was high but variable due to the mixed background of the mice. Diabetes did not significantly increase GBM thickness or podocyte foot process width (fig 4G&H). However, podocyte slit diaphragm narrowing was evident in D-LMC mice (fig 4G&H). D-LMC mice had significantly lower endothelial fenestration density than LMC, whereas D-podVEGFC did not (fig 4G&I).

*Glomerular VEGFC intervention reduces the development of DN*



To understand whether VEGFC could rescue DN after diabetes was established, doxycycline was administered 4wk post STZ injections (fig 5A). Diabetic mice were hyperglycemic (fig 5B) and polyuric (fig 5C) as anticipated. There was no significant difference in weight gain between groups, however, these diabetic mice were susceptible to intestinal bloating which was likely to mask body weight reductions (this protocol was carried out chronologically before the one described above, and this issue was mitigated as described in the methods) (fig 5D). Of note, this cohort of mice also had a lower starting weight than the intervention cohort in fig 4, and sham mice did not gain weight as rapidly which may confound the results. Elevated plasma uric acid is associated with diabetes and can be caused by hyperglycemia (46). Plasma uric acid was significantly raised in both diabetic groups as anticipated (fig 5E). Importantly, renal hypertrophy (fig 5F) was significantly increased in D-LMC mice, but significantly reduced in D-podVEGFC mice. Fold change in uACR (fig 5G, please refer to supp fig 5B for absolute values) was significantly increased in D-LMC mice but not in D-podVEGFC mice. Mesangial matrix expansion was significantly reduced in D-podVEGFC mice (fig 5H&K), supported by decreased collagen deposition (Fig 5I, J). There was no significant effect of diabetes on GBM thickness, consistent with an early phenotype of DN (fig 6A-B). D-LMC had significantly increased podocyte foot process width and both D-LMC and D-podVEGFC had significantly reduced slit diaphragm width (fig 6B). D-LMC also had significantly reduced fenestration density (fig 6C) demonstrating the early development of diabetic nephropathy. Together, these data from both diabetic cohorts suggest that early VEGFC intervention may reduce the development of early DN.

*Glomerular VEGFC prevents the diabetes associated changes in VEGFR expression*

Induction of VEGFC expression was confirmed in glomerular fractions from D-podVEGFC mice (fig 7A) in a similar manner to podVEGFC mice. There was no significant change in endogenous glomerular fractions of VEGFC expression between D-LMC and sham-LMC mice (fig 7B). VEGFA mRNA from glomerular fractions was increased by diabetes (fig 7C), as previously shown (47), with a trend towards increase in D-podVEGFC mice (fig 7C). VEGFR2 and VEGFR3 mRNA from glomerular fractions were significantly increased in D-LMC mice, but not D-podVEGFC (fig 7D-E). This was supported by VEGFR2 protein analysis whereby VEGFR2 expression was significantly reduced in D-podVEGFC mice (fig 7F, G). Of note, VEGFR2 protein was undetected in sieved flow-through fractions (supp fig E), suggesting that VEGFR2 protein changes were glomerular specific. VEGFR3 protein analysis was not significant (fig 7F, H). In summary, VEGFC prevented VEGFR changes associated with DN.

*VEGFC protects against raised Ps'alb caused by systemic eGLX dysfunction*

We have previously shown that VEGFC increases the synthesis of sulphated and non-sulphated glycosaminoglycans (GAG) in GEnC-GLX *in vitro* (33). Since macromolecular barrier properties in GEnC are governed by eGLX (48) we investigated the potential for VEGFC to reduce Ps'alb in an eGLX dependent manner. Previously, we demonstrated that a bolus of hyaluronidase and chondroitinase 30min before sacrifice significantly reduced eGLX coverage and significantly increased Ps'alb(40) . In the same cohort of mice, isolated glomeruli were stimulated with VEGFC for 1hr. In these glomeruli, VEGFC rescued the effect of eGLX disruption on Ps'alb (fig 8A). We followed this experiment with chronic, systemic exposure to these enzymes (2wk) with VEGFC i.p. injections given in the last week. Enzymes significantly increased Ps'alb in isolated glomeruli. However, this was significantly prevented in glomeruli from mice with given the VEGFC intervention (fig 8B). Electron micrographs from mice in

parallel experiments demonstrated that GAG enzymes significantly reduced GEnC eGLX depth, but not in the presence of VEGFC (fig 8C-D). Interestingly podocyte glycocalyx was relatively unaffected (fig 8D). To demonstrate no off-target effects of the GAG enzymes, glomerular ultrastructural parameters were measured. There were no significant effects of enzymes on GBM width (although the combination of enzymes plus VEGFC significantly reduced GBM width), slit diaphragm width, podocyte foot process density (fig 8E, although there was a significant overall effect) or endothelial fenestration density (fig 8F). In summary VEGFC can protect from increased Ps'alb under eGLX dysfunction, suggesting the possibility that VEGFC protection from albuminuria is eGLX dependent.

## Discussion

In this study, we demonstrate for the first time that VEGFC can protect against VEGFA-induced glomerular albumin permeability and can restore glomerular albumin permeability in type II diabetes. Also, it can ameliorate early DN in type I diabetes; reducing fibrosis, kidney hypertrophy, loss in glomerular endothelial fenestration density, and reducing albuminuria. There was an 89% reduction in albuminuria when VEGFC was used preventatively and a 67% reduction when VEGFC was used to rescue changes. These were comparable to previously published results demonstrating that glomerular over-expression of angiopoietin-1 in diabetic mice caused a 70% reduction in albuminuria (49) and glomerular over-expression of VEGFA<sub>165b</sub> in diabetic mice caused an 80% reduction in albuminuria (50). That VEGFC reversed the increased glomerular albumin permeability in type II diabetic glomeruli confirms the effect of VEGFC across mouse strains and diabetes type. This demonstrates that VEGFC can act as an anti-albuminuric agent in experimental DN.

Diabetes-induced damage to glomerular ultrastructure included foot process flattening and reduced endothelial fenestration density (51-53). Reduced fenestration density is an established feature of DN in humans, which is positively correlated with uACR (52). It has been suggested that loss of GEnC fenestration density reflects both glomerular filtration rate and uACR better than changes in podocyte parameters (54). Of note we did not see any changes in GBM thickness. This is surprising, but may be a reflection that podocyte damage is not yet severe enough to impact maintenance of the GBM (55), or may simply be a reflection of mixed strain background. Notably the D-podVEGFC mice did not have significantly reduced fenestration density. It is well established that GEnC fenestration density is reduced in DN. Fenestrations are too large to restrict albumin passage, however loss of fenestration density, or organization, may

reflect a loss of filtration control. Also, renal hypertrophy was reduced in D-podVEGFC mice. Although our mice have early diabetic nephropathy they also have evidence of fibrosis, demonstrated by glomerular PAS staining and increased glomerular and tubular collagen I and III deposition, as is predicted in diabetes (56). Kidney weight is partially reduced in the D-podVEGFC mice. This may indicate a reduction in early fibrosis as a result of reduced albumin filtration and therefore reduced tubular toxicity (57).

We suggest the differing effects between VEGFC and VEGFA may be due to differential activation of VEGFR2. We have previously shown that VEGFC does not induce VEGFR3 phosphorylation in ciGenC, yet promotes delayed VEGFR2 phosphorylation at 30min compared to rapid phosphorylation by VEGFA at 2min (32). However, we show that VEGFC causes rapid loss of cell surface VEGFR3 expression, suggesting internalisation of VEGFR3 by VEGFC. In otherwise healthy glomeruli, chronic glomerular VEGFC expression induced VEGFR2 and VEGFR3 heterodimerisation and reduced glomerular VEGFA mRNA expression without inducing glomerular fibrosis or a loss of glomerular function or changes in glomerular ultrastructure. This is important since reduced glomerular VEGFA expression can lead to renal thrombotic microangiopathy and proteinuria (26; 44), yet increased glomerular VEGFA expression can lead to collapsing glomerulopathies and proteinuria (44). That VEGFC has no detrimental effect on glomerular function is fundamentally important to future studies aiming to manipulate the VEGFC pathway in order to protect glomeruli. It is known that VEGFC induces VEGFR2/VEGFR3 heterodimerisation in lymphatic endothelial cells (45). Here, we demonstrate that chronic exposure to VEGFC induces heterodimerisation in glomeruli. Also, glomerular VEGFA expression is repressed, similarly to when Ang-1 is overexpressed (58), potentially altering receptor availability to VEGFC and thus facilitating heterodimerisation. Interestingly,

heterodimerisation of VEGFR3 with VEGFR2 leads to reduced phosphorylation of some tyrosine sites in lymphatic endothelial cells (45). These results suggest that VEGFR3 is involved in VEGFC signalling, confirm functional activity of overexpressed VEGFC and demonstrate for the first time *in vivo* that mouse VEGFC induces VEGFR2/VEGFR3 heterodimerisation. Thus, the change in glomerular VEGFR complex formation may explain the different temporal effects on VEGFR2 phosphorylation by VEGFA and VEGFC. Interestingly, VEGFC also protected from a diabetes-induced increase in VEGF receptor expression in glomeruli. These data support previous observed increases in VEGFR2 expression in DN (59; 60), although changes in glomerular VEGFR3 expression are not well documented. VEGFR2 and VEGFR3 expression were both reduced in D-podVEGFC mice, therefore the potential for heterodimerisation remained the same. However, the potential for receptor *phosphorylation* may be reduced, thereby reducing downstream signalling. Glomerular VEGFA is known to be upregulated in early DN (18) and most glomerular VEGF-receptor complexes are localized to GEnC (18). This indicates that VEGFA predominantly regulates the endothelial phenotype during diabetic glomerulopathy, although endothelial cells are thought to “signal back” to the podocytes, inducing their injury (44). Hohenstein et al demonstrated that mildly injured glomeruli in early DN (relating to glomeruli in our study) had increased VEGFA-bound receptor complexes, whereas more severely injured glomeruli had decreased VEGFA-bound receptor complexes (18). Perhaps the key for VEGFC-mediated protection is to competitively reduce VEGFA receptor signalling, particularly in early DN.

VEGFC induces lymphatic hyperplasia and is associated with lymphangiomas and lymphangiosarcomas (61). Subcutaneous VEGFC overexpression also increases obesity and insulin resistance (62) and VEGFC is implicated in inflammation in late DN (63). Therefore, it is

unlikely that VEGFC can be given therapeutically in its native form, although, of note, it was used successfully in mice to treat polycystic kidney disease (64), to treat renal interstitial fibrosis for 14 days (65) and we gave VEGFC daily i.p for a total of 7 days with no gross morphological effects. Lymphangiogenesis is increased in the tubular-interstitium in DN (63) and this is considered a protective mechanism to aid drainage (66), but may also promote inflammation (67). If this mechanism is saturated, it can lead to oedema in the kidney, as suggested by (66). Importantly, in a model of unilateral ureteral obstruction, VEGFC treatment by osmotic mini-pump attenuated infiltration of inflammatory cells (65). We have not yet investigated whether lymphangiogenesis is promoted in podVEGFC mice, but this is an important consideration in diabetes and is another potential reason for reduced hypertrophy in podVEGFC diabetic mice. Whether VEGFC can act as a microvascular protectant in other vascular beds in diabetes remains to be seen. In order to harness the effects of VEGFC therapeutically it may be more appropriate to target the VEGFC signalling pathway. One such approach would be to target the VEGFR2/VEGFR3 complex as previously for VEGFR1/VEGFR2 complexes (68). Another would be to target signalling pathways that promote eGLX synthesis. If VEGFC can protect from diabetes-induced glycocalyx damage, then this is another exciting avenue of treatment. We have shown that VEGFC can protect against increased Ps'albumin caused by systemic eGLX damage, both in chronic and acute models. We have also previously shown *in vitro* that VEGFA induced eGLX shedding in GEnC whilst VEGFC induced eGLX synthesis (33). As highlighted above, the D-podVEGFC mice did not suffer from significant fenestration density loss. The restoration of filtration control for albumin is potentially due to altered e-GLX coverage of the fenestrations. Since eGLX dysfunction is considered an early insult in DN (69), and the type I diabetes models

used show an early phenotype, perhaps VEGFC mediates its protection through eGLX restoration in early DN. This will be a focus of future work.

Importantly, we have demonstrated a novel role for VEGFC in the development of early DN.



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KLO helped design experiments, acquired data, interpreted results and approved the final version. MG, NRB, SLB, KBB, SD, and RR, all acquired data interpreted results and approved the final version. CRN advised on EM imaging and measurements and approved the final version. PWM, LG, KA, DOB, AJS, GIW and SCS helped design experiments, interpreted results and approved the final version. RRF, the guarantor who takes full responsibility for the work as a whole, conceived and designed the work, interpreted the results, revised the manuscript and approved the final version.

## **Disclosures**

No authors have any competing financial interests. Dr R Foster is the Guarantor for this work.

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## Figure legends

**Figure 1.** VEGFC prevents the increase in glomerular albumin permeability caused by VEGFA and type II diabetes. Glomeruli were sieved from healthy mice (FVB/mixed background (**A**) and db/+ lean control (**B**)) and stimulated *ex vivo* with vehicle, 1nM VEGFA, 10nM VEGFC or both for 1hr at 37°C and Ps'alb measured (one-way ANOVA with Bonferroni post hoc tests indicated). Glomeruli were sieved from db/+ and db/db mice at 12-14wk and incubated *ex vivo* with (+) or without (-) VEGFC, then Ps'alb measured (**C**, one-way ANOVA, Bonferroni post hoc tests indicated). Number of animals indicated outside brackets and number of glomeruli indicated inside brackets. UACR (**D**) and blood glucose (**E**) are shown for db/+ and db/db mice at 12-14wk. Fold change in VEGFA mRNA expression in sieved glomeruli from db/+ and db/db mice is shown (**F**, n= 6 and 5 respectively). \*= $p<0.05$ , \*\*= $p<0.01$ , \*\*\*= $p<0.001$ .

**Figure 2.** Characterization of podVEGFC mouse. **A.** A 2.5-kb fragment of the NPHS2 promoter-enhancer region directs the expression of reverse tetracycline-controlled transcriptional activator rtTA in podocytes. In the presence of doxycycline, a transcriptional activator fusion protein binds to the tetracycline-response operon promoter element (TetO) and initiates mouse VEGFC transcription (29). **B.** Glomeruli were isolated from podVEGFC (podVC) or LMC (littermate control) mice after receiving doxycycline for 3 wk. mRNA was extracted and QPCR performed for VEGFC, normalised to GAPDH and to LMC animals ( $2^{-\Delta\Delta CT}$ ) (\*= $p<0.05$ , unpaired t-test, n=3). **C.** Kidneys from LMC and podVEGFC mice on doxycycline for 8 weeks were removed, snap frozen and co-immunofluorescence was performed with anti-VEGFC (green), anti-nephrin (red) antibody and DAPI (blue). Representative confocal images are shown. Images are shown at 10x (i, scale bar = 25µm) and x40 (ii, Scale bar= 70µm). **D.** Semi-quantification was performed on glomeruli (VEGFC/Dapi) and presented as fold change ( $p<0.05$ , unpaired t-test, n=9 and 5

respectively). Kidneys from LMC and podVEGFC mice given doxycycline for 8 wk were weighed and expressed as a ratio to total body weight (**E**). Periodic acid Schiff's (PAS) staining and ultrastructural changes were imaged (**Fi** and **Fii**). PAS staining was quantified (**G**, n=5&8) as were parameters of glomerular ultrastructure, including glomerular basement membrane (GBM) width, podocyte foot (FP) process width, slit diaphragm (SD) length (**H**, n=4&8) and fenestration density per  $\mu\text{m}$  length GBM (**I**, n=4&8). Weekly urine albumin creatinine ratios (uACR) are expressed (**J**, n=6&5). N.s. = non-significant.

**Figure 3.** VEGFC overexpression affects VEGFA and VEGF receptors. Glomeruli were enriched from podVEGFC and littermate control (LMC) mice. Quantitative PCR was performed and normalised to GAPDH and LMC mice ( $2^{-\Delta\Delta\text{CT}}$ ). Relative VEGFR2 (**A**), VEGFR3 (**B**) and VEGFA (**C**) mRNA expression is shown (n=3.  $\ast=p<0.05$ , unpaired t-test). Human GEnC were stimulated with VEGFC or VEGFA for 2min then fixed, non-permeabilised or permeabilised and stained for VEGFR3 or IgG control (scale bar 50 $\mu\text{m}$ ). Representative images are shown (**Di**). Fold change in mean fluorescent intensity of VEGFR3 per cell is shown for non-permeabilised (**Dii**) and permeabilized (**Diii**) GEnC (n=5-6,  $p<0.001$  one-way ANOVA). Isolated glomeruli (**E**) and homogenised lung (**F**) from wild type mice were treated *ex vivo* with vehicle, VEGFC (30min) and VEGFA (2min, glomeruli fractions only). Samples were immunoprecipitated with anti-VEGFR3 then probed with anti-VEGFR3 or anti-VEGFR2. These are representative blots from 3 separate experiments. A proximity ligation assay was performed on fresh frozen kidney sections from LMC or podVEGFC mice using antibodies to VEGFR2 and VEGFR3 (**G**, scale bar 25 $\mu\text{M}$ ). Punctate events were counted through Z stacks in glomeruli (outlined) and expressed per nuclei (**H**).  $\ast=p<0.05$ , unpaired t-test, n=6 and 3.

**Figure 4.** Early VEGFC expression reduces the development of DN. Mice were given doxycycline at the same time as STZ injections, at wk0 (**A**). Glycemia (**B**), body weight (**C**) urine output (**D**), kidney/body weight ratio (**E**,  $p<0.05$ , one-way ANOVA) were monitored weekly post injection in D-LMC (diabetic) and D-podVEGFC mice compared to sham-LMC mice ( $n=11, 3 \text{ \& } 5$ ). Log fold change to baseline in uACR is shown at 8wk post STZ injection (**F**,  $p<0.05$ , one-way ANOVA,  $n=11, 11 \text{ \& } 5$  respectively). Mice were cardiac perfusion fixed. Representative electron micrographs are shown at high power to show details of capillary loops in sham-littermate control (LMC) (**Gi**), diabetic LMC (D-LMC) (**Gii**) and diabetic podVEGFC (D-podVEGFC) (**Giii**). Arrows highlight reduced endothelial fenestration density and arrowheads highlight flattened foot processes. Ultrastructural changes were quantified by measuring GBM width, podocyte foot process width ( $p<0.05$ , one-way ANOVA) and slit diaphragm (SD) length ( $n= 7, 4 \text{ \& } 5$  respectively,  $p<0.001$ , one-way ANOVA, **H**). Endothelial fenestration density per  $\mu\text{m}$  of GBM was also quantified ( $n= 7, 4\&5$ ,  $p<0.05$ , one-way ANOVA, **I**). Bonferroni post hoc tests indicated;  $*=p<0.05$ ,  $**=p<0.01$ ,  $***=p<0.001$ ,  $n=5, 3\&4$ . D-podVC=D-podVEGFC.

**Figure 5.** VEGFC intervention reduces the development of DN.. Mice were given STZ injections at wk 0, then given doxycycline after the establishment of hyperglycemia at wk 4 (**A**). Glycemia (**B**,  $n=4, 20 \text{ \& } 9$ ), urine output (**C**,  $n=4, 16 \text{ \& } 11$ ) and body weight (**D**,  $n=4, 20 \text{ \& } 9$ ) were measured weekly in sham-LMC, diabetic littermate control (D-LMC) and diabetic podVEGFC (D-podVC) mice. Plasma uric acid (**E**,  $p<0.05$ , one-way ANOVA), kidney:body weight ratio (**F**,  $p<0.05$  one way ANOVA,  $n=4, 10\&9$ ) and urine albumin creatinine ratios (uACR), presented as log fold change to baseline (**G**,  $p<0.05$ , one way ANOVA,  $n=5, 18 \text{ \& } 15$ ) were also measured at 8 wk post injection. Representative Periodic acid Schiff's (PAS) staining



was performed on paraffin embedded kidney sections from sham-LMC, D-LMC and D-podVEGFC mice (**H**). Representative picrosirius red immunohistochemistry images are demonstrated under brightfield (**I**) and polarized (**J**) lens (n=3). PAS staining intensity is quantified using Quantity One (**K**,  $p<0.001$ , one-way ANOVA, n=3). Post hoc tests indicated,  $*=p<0.05$ ,  $**=p<0.01$ ,  $***=p<0.001$ .

**Figure 6.** VEGFC intervention ameliorates early ultrastructural changes in DN. Mice given doxycycline at 4wk post STZ were cardiac perfusion fixed. Representative electron micrographs are shown at high power to show details of capillary loops in sham-littermate control (LMC) (**Ai**), Diabetic-LMC (D-LMC) (**Aii**) and D-podVEGFC (D-podVC) (**Aiii**). Arrows highlight reduced endothelial fenestration density and arrowheads highlight flattened foot processes. Ultrastructural changes were quantified by measuring GBM width, podocyte foot process width ( $p<0.05$ , one-way ANOVA) and slit diaphragm (SD) length (n= 4, 6 & 3 respectively,  $p<0.001$ , one-way ANOVA, **B**). Endothelial fenestration density per  $\mu\text{m}$  of GBM were also quantified ( $p<0.05$ , one-way ANOVA, **C**). n=4, 6 & 3. Bonferroni post hoc tests indicated;  $*=p<0.05$ ,  $**=p<0.01$ ,  $***=p<0.001$ .

**Figure 7.** Glomerular VEGFC prevents the diabetes associated changes in VEGFR expression. Glomeruli enriched fractions from sham littermate control (LMC), diabetic podVEGFC (D-podVC) and littermate control (D-LMC) mice given doxycycline from 4wk post injection. mRNA was extracted and quantified using qPCR normalised first to GAPDH then LMC ( $2^{-\Delta\Delta\text{CT}}$ ). Glomerular enriched fraction VEGFC expression is demonstrated by QPCR in D-podVEGFC compared to D-LMC mice (i.e. overexpressed) (**A**,  $p<0.05$ , unpaired t-test, n=4) and in D-LMC compared to sham mice (i.e. endogenous) (**B**, n=4). Glomerular enriched fraction

VEGFA mRNA expression is demonstrated by QPCR in sham-LMC, D-LMC and D-podVEGFC mice (**C**,  $p<0.05$ , one-way ANOVA,  $n=3$ ). Glomerular VEGFR2 (**D**,  $p<0.01$ , one-way ANOVA,  $n=3$ , 3 & 4) and VEGFR3 (**E**,  $p<0.05$ , one-way ANOVA,  $n=3$ , 3 & 4) expression in sham-LMC, D-LMC and D-podVEGFC mice are shown. Expression changes at the protein level for VEGFR3 and VEGFR2 were confirmed by Western blotting in isolated glomeruli from the same groups of mice. VEGFR2 overexpressing GEnC (GEnC-R2) and mouse spleen were used as positive controls for VEGFR2 and VEGFR3 respectively (**F**,  $n=3$ ). Normalised density to actin, then sham for VEGFR2 (**G**,  $n=3$ ,  $p<0.01$ ) and VEGFR3 (**H**,  $n=4$ , 6&3 respectively) is shown. Bonferroni post hoc tests indicated,  $*=p<0.05$ ,  $**=p<0.01$ ,  $***=p<0.001$ .

**Figure 8.** VEGFC protects against raised Ps'alb caused by systemic eGLX dysfunction. FVB/mixed background mice were administered hyaluronidase and chondroitinase either acutely (30min i.v. **A**) or chronically (osmotic minipump, 2wk **B-F**). Glomeruli isolated from mice with acute eGLX dysfunction (enzymes +) were incubated *ex vivo* with (+) VEGFC, then Ps'alb measured and compared to historical data without (-) VEGFC (grey(40) ) (**A**, one-way ANOVA with Bonferroni post hoc tests indicated). Mice exposed to enzymes chronically over a period of 2wk were given VEGFC i.p in the last week. Ps'alb measurements were taken in isolated glomeruli (**B**, one-way ANOVA, Bonferroni post hoc tests indicated). Representative EM images of the glomerular filtration barrier for each condition ( $n=4$ , 3, 4 respectively) are shown with e-GLX highlighted (white brackets, **C**, scale bar 100nm). Glomerular ultrastructural parameters from these mice were quantified; eGLX depth, podocyte GLX depth (**D**, one-way ANOVA with Bonferroni post hoc tests indicated, n.s. = non-significant), glomerular basement membrane (GBM) thickness (one way ANOVA, Bonferroni post-hoc tests indicated), podocyte foot process (FP) width, slit diaphragm (SD) length (**E**,  $p<0.05$ , one way ANOVA, no post hoc test

significance) and endothelial fenestration density (**F**).  $\ast=p<0.05$ ,  $\ast\ast=p<0.01$  (dashed lines indicate comparison of previously published data)  $\ast\ast\ast=p<0.001$ .

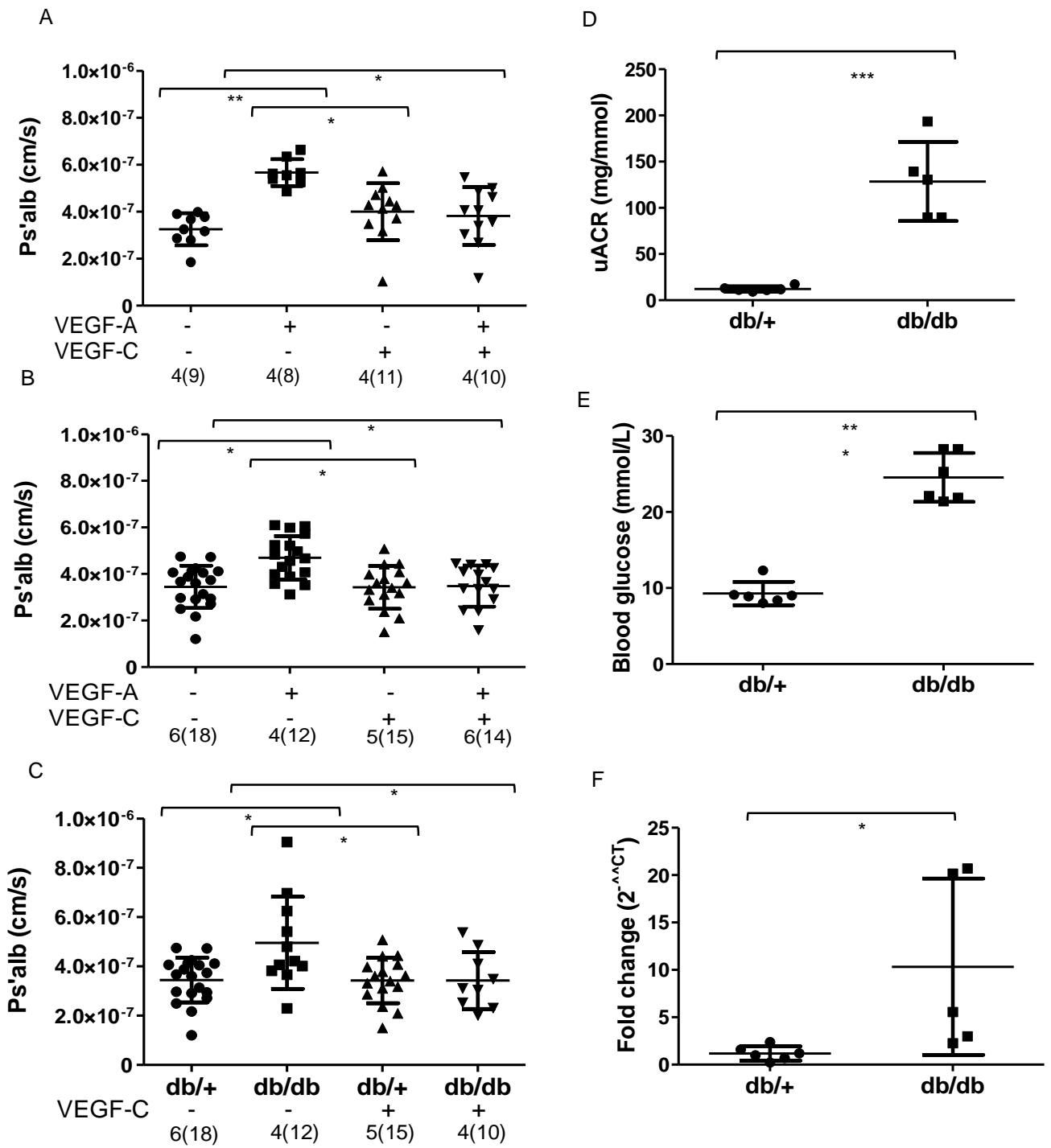


Figure 1.

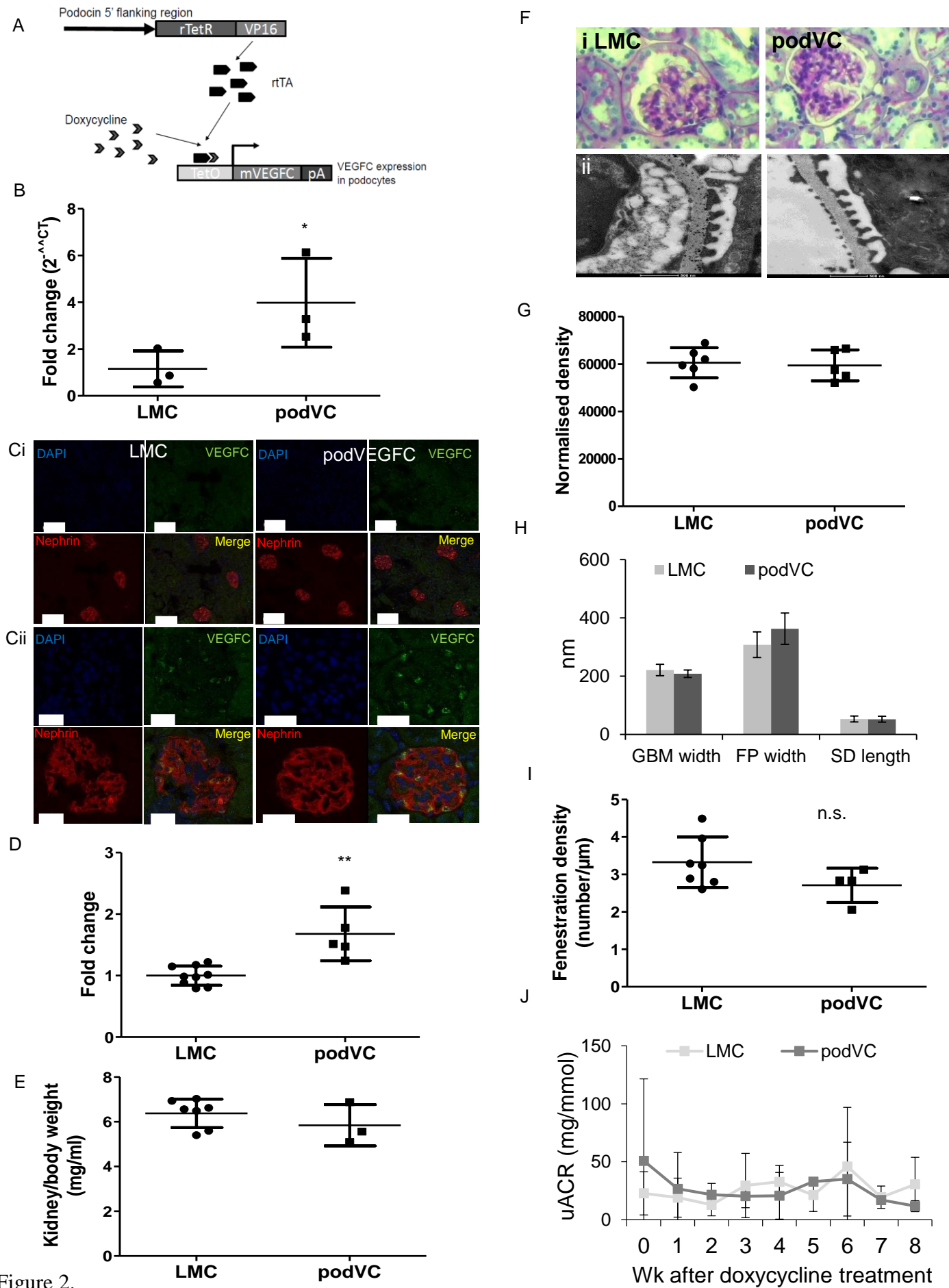


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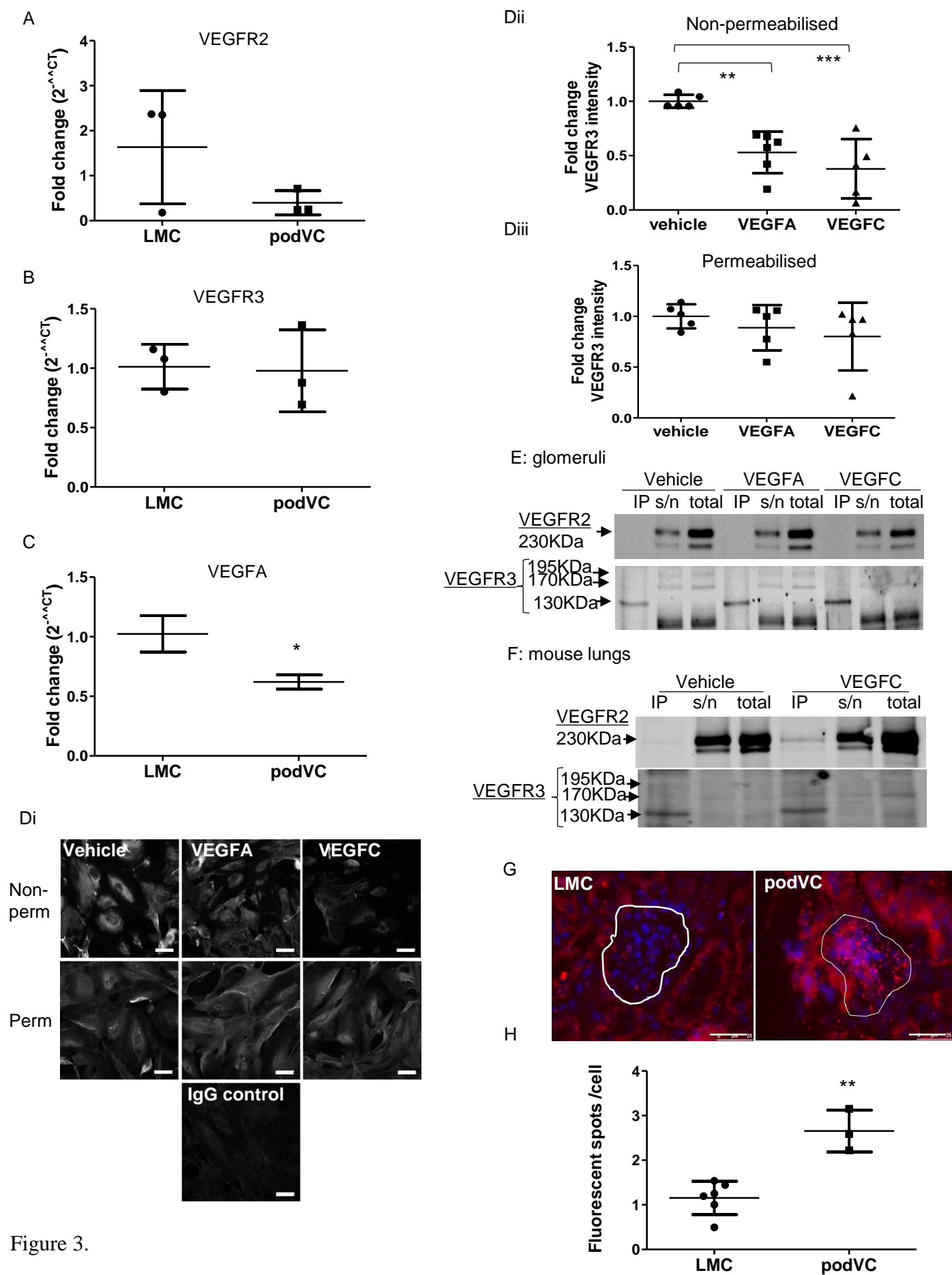


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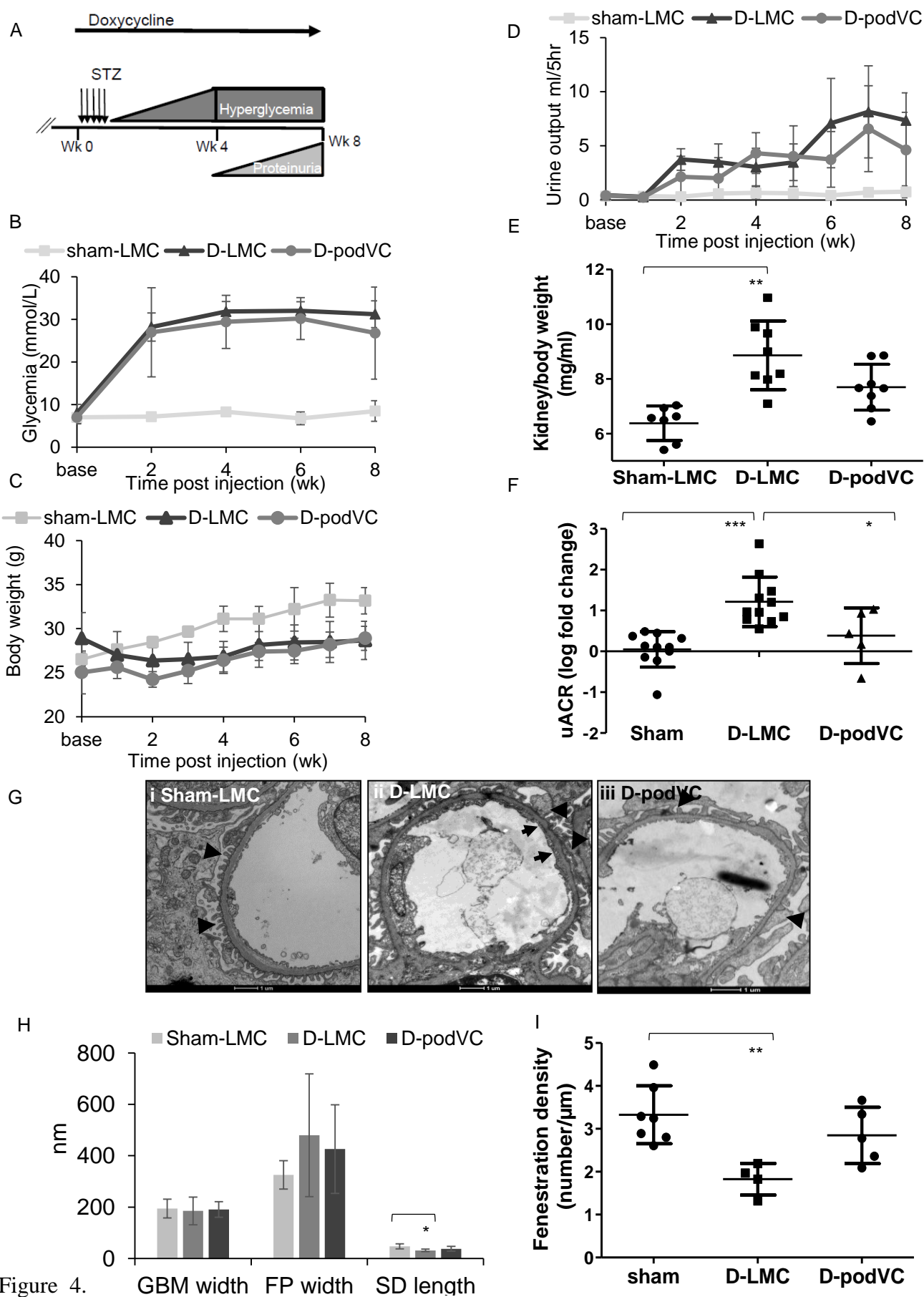


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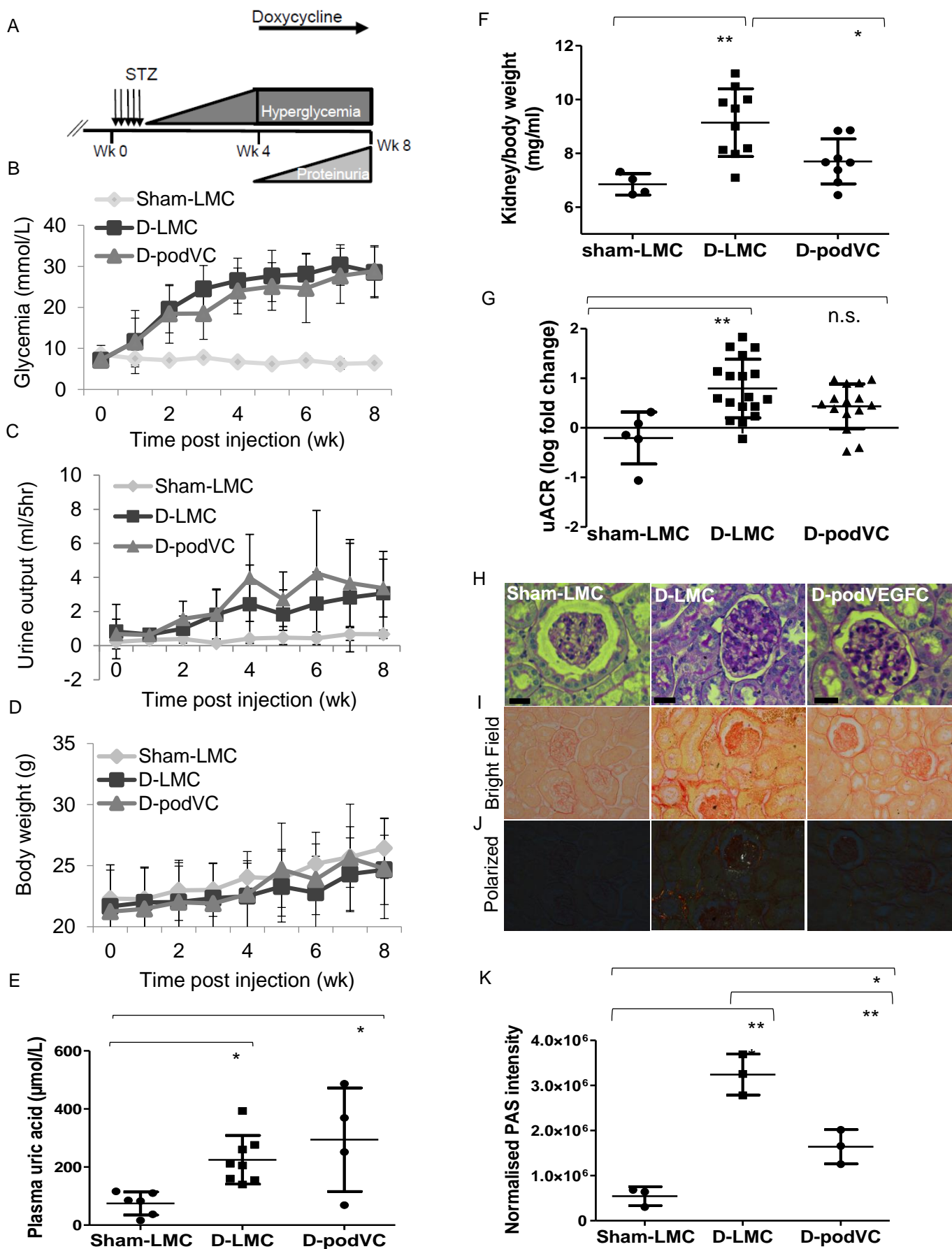
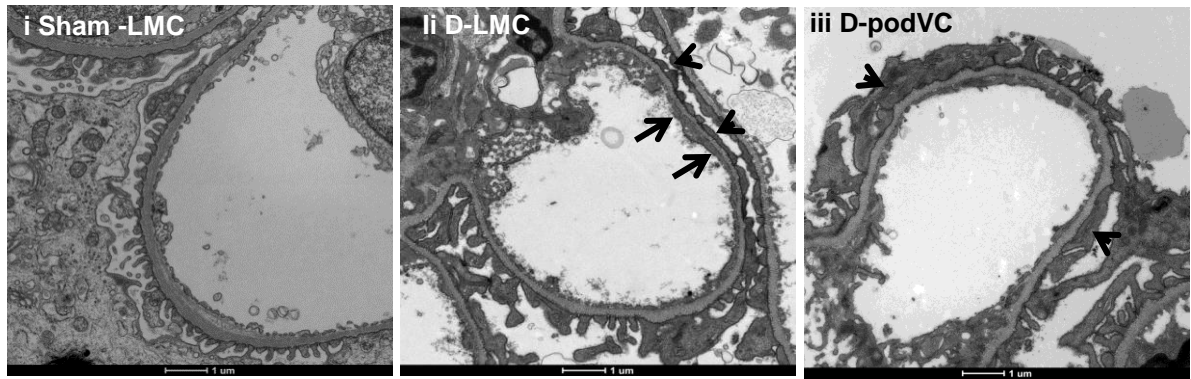


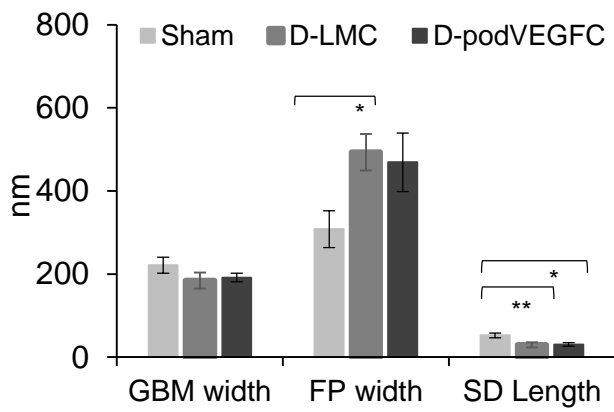
Figure 5.



A



B



C

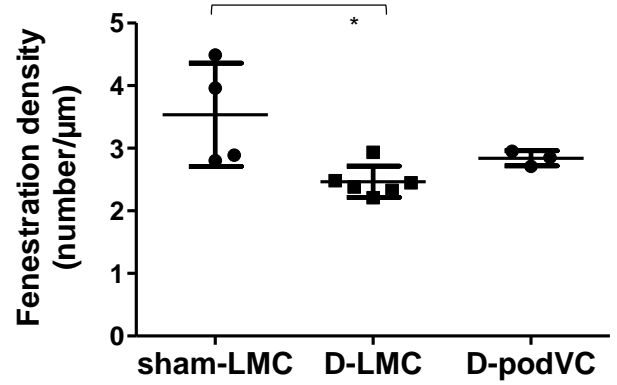


Figure 6.

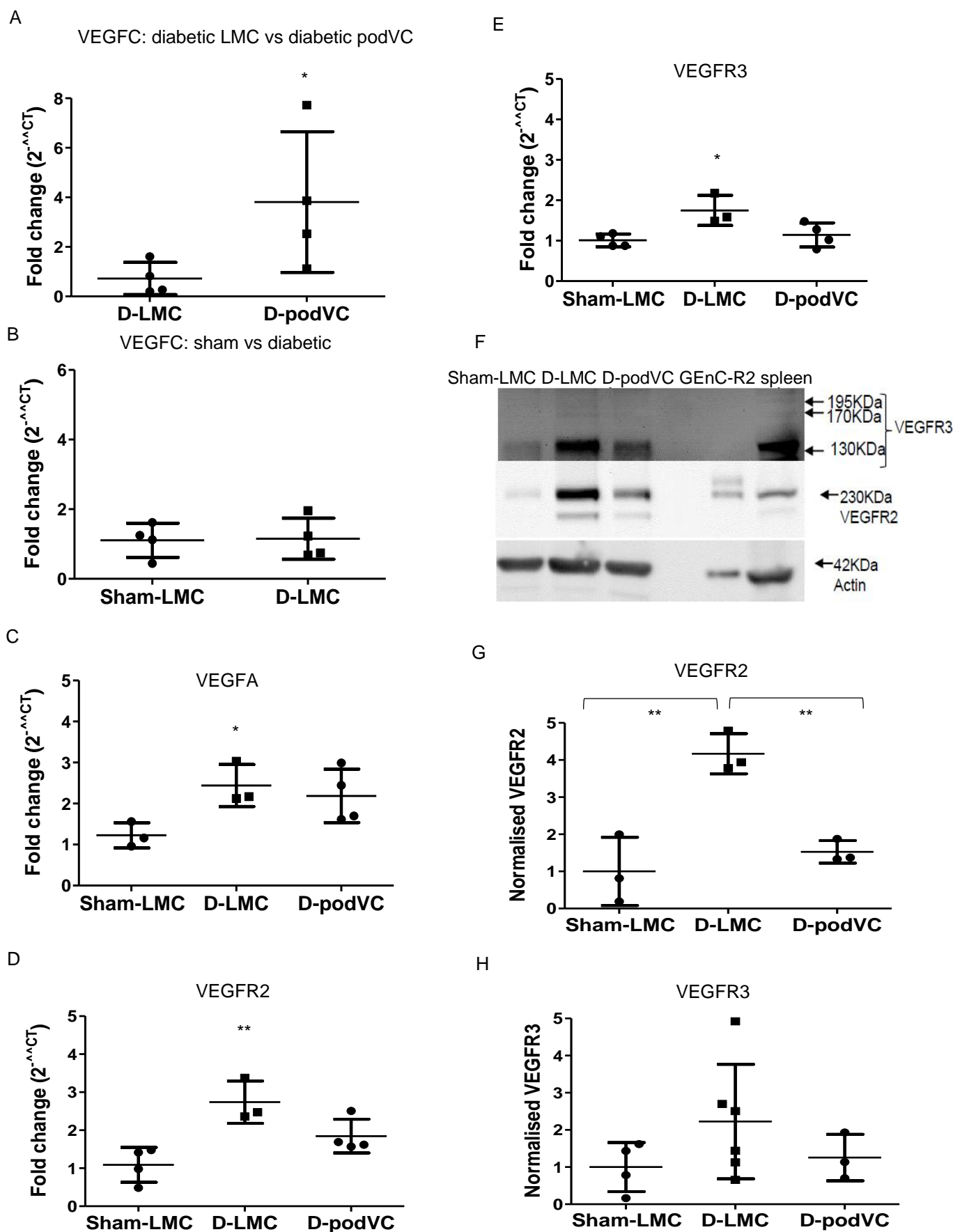


Figure 7.

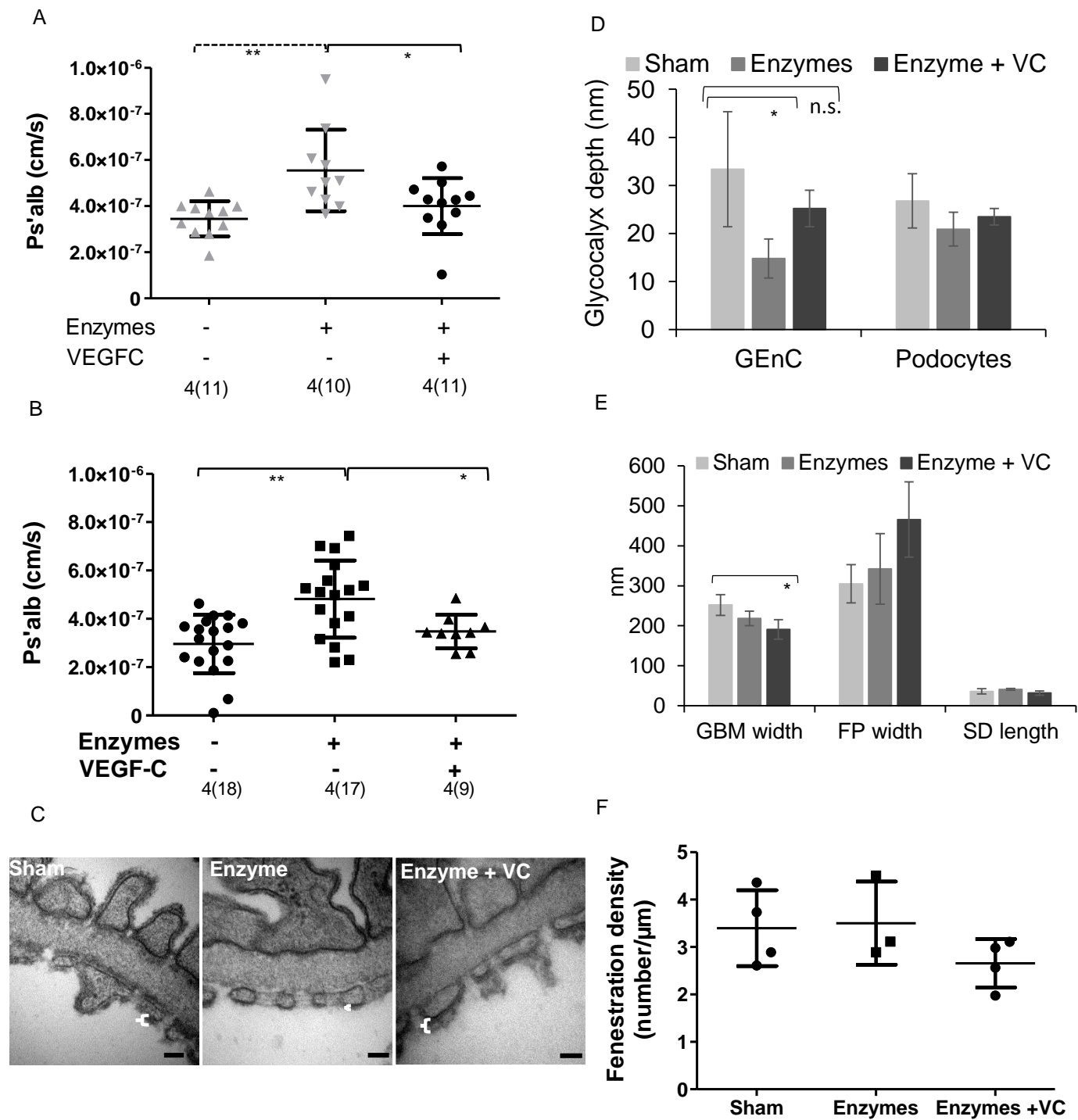


Figure 8.